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Effect of Brain on Proliferative Activity of Adenohypophysial Primordial Cells *in vitro*

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ABSTRACT—The pattern of cell proliferation in the fetal rat adenohypophysial primordium was investigated both in vivo and in vitro with special reference to the diencephalic floor. Proliferating cells were labelled by bromodeoxyuridine (BrDU) followed by its immunohistochemical detection with a monoclonal antibody. In fetal rats of 12.5 days of age, BrDU-labelled cells were distributed almost evenly throughout the adenohypophysial primordium. On days 13.5 and 14.5, on the other hand, labelling was mostly confined to the dorsal wall of the adenohypophysial primordium that was in contact with the diencephalic floor. To examine the *in vitro* effect of the developing diencephalic floor on cell proliferation of the adenohypophysial primordium, Rathke's pouches were isolated from fetal rats on days 12.5 and 13.5 of gestation and kept in organ culture for 1–2 days with or without the brain. The incidence of BrDU-labelled cells was markedly high if the diencephalic floor was left intact. Moreover, proliferating adenohypophysial primordial cells were concentrated in the region adjacent to brain tissue. In the absence of brain, only a small number of cells were labelled. Such a low incidence of labelling was also observed when brain was replaced by liver. From these results we conclude that the developing diencephalon is essential for proliferation of adenohypophysial primordial cells. It remains to be settled if the adoral part of primordial cells can also respond to this neural agent.

INTRODUCTION

The hypophysis consists of two different components, i.e., neuro- and adenohypophysis. From the early stage of development, the adenohypophysial primordium makes close contact with the diencephalic floor. Such a close relationship between the two tissues has lead many investigators to assume that the differentiation of the adenohypophysis is under the influence of the brain. In amphibians, the pars intermedia fails to form after removal of the posterior hypothalamus [1–4]. The reports are contradictory as to whether brain is essential for the development of the pars distalis: some workers accept the inductive influence of the brain [5, 6], whereas others believe that neural tissue plays little role [1, 3, 4]. In mammals, we have previously shown the involvement of the diencephalic floor in the cytodifferentiation of the pars distalis *in vitro* [7–9]. Moreover, cell proliferation of adenohypophysial primordium also appeared to be stimulated by brain since its removal caused a marked reduction in the size of culture explants. Daikoku et al. [10] have provided quantitative data that the size of the adenohypophysial primordium markedly increased when it was co-cultivated with brain. *In vivo,* mitotic figures are more frequently observed in the dorsal region of the adenohypophysial primordium that was in contact with the diencephalic floor [11, 12]. Recently, Ikeda and Yoshimoto [13] have studied proliferative activity of the fetal rat hypophysis by use of bromodeoxyuridine (BrDU). According to their report, BrDU-labelled cells were concentrated in the dorsal portion of the adenohypophysial primordium that faces the developing neural lobe. To date, however, there is little direct information on the role of the neural element. In this study we examined the *in vitro* effect of diencephalic floor on cell proliferation of the adenohypophysial primordium. In addition, we re-investigated the pattern of cell proliferation in the rat adenohypophysial primordium *in vivo.*

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MATERIALS AND METHODS

Adult rats of the Sprague-Dawley strain were mated at night. If spermatozoa were found in the vaginal smears the next morning, the noon of that day was designated at day 0.5 of gestation.

In vivo experiments

Pregnant rats from day 12.5 to 16.5 of gestation received an intraperitoneal injection of bromodeoxyuridine solution (30 mg/kg, Amersham, UK). Three hours after injection, mothers were anesthetized with ketamine hydrochloride and fetuses were removed for fixation. Heads were immersed in Bouin's solution and trimmed under a dissecting microscope to obtain good penetration of fixative into the adenohypophysial primordium.

In vitro experiments

Pregnant rats of days 12.5 and 13.5 were anesthetized and fetuses were removed one by one by Caesarian section. The hypophysial primordium was separated in Ca- and Mg-free Hanks solution as described previously [14]. In some primordia mesenchymal tissue was removed as much as possible with the diencephalic floor left intact. These were cultivated so as to adenohypophysial and neural tissue as well as their boundary were clearly shown during the subsequent histological processes (Fig. 1). In other primordia the diencephalic floor and mesenchyme were removed by the use of 0.15% collagenase (Sigma, type V) in Ca- and Mg-free Hanks solution. During this period, the upper half of the primordium was in contact with the developing brain, whereas its lower half was surrounded by a rich amount of mesenchyme. A small number of mesenchymal cells were also observed between the adenohypophysial primordium and future infundibulum. Removal of the latter mesenchyme was impossible without separating the brain. Those primordia from which only mesenchyme was removed in the enzyme solution served as a control. After enzymatic removal of the brain from a few adenohypophysial primordia, they were combined with a piece of the hepatic rudiment. In this study enzyme treatment was completed as quickly as possible, generally within 10 min. Tissues were then placed on pieces of cellulose acetate membrane and maintained in Falcon dishes (no. 3037) for organ culture. The culture medium was α-MEM to which 30 mM glucose was added. Fetal bovine serum (Gibco) was added at a concentration of 0.1%; this low concentration was employed to minimize the effects of growth factors, if any, contained in the blood serum. From 1 to 2 days after cultivation, BrDU solution was added at a dose of 6 μg/ml medium. Explants were fixed in Bouin's solution 3 hr later. After overnight fixation, they were embedded in paraffin and cut at 2 μm using glass knives. Deparaffinized sections were first incu-

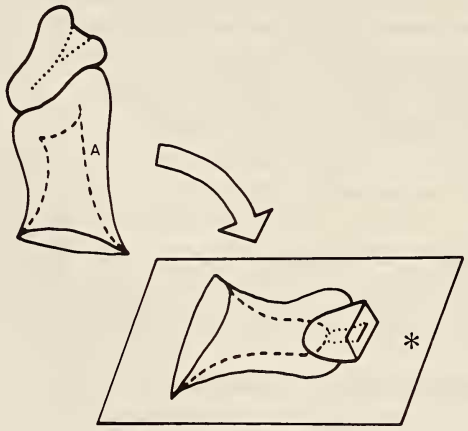

Fig. 1. Diagram showing how the adenohypophysial primordium was placed on a piece of cellulose acetate membrane. The primordium was oriented with its anterior wall (A) at the bottom. Asterisk indicates a mark which was made in order to facilitate the identification of brain tissue at the time of fixation.

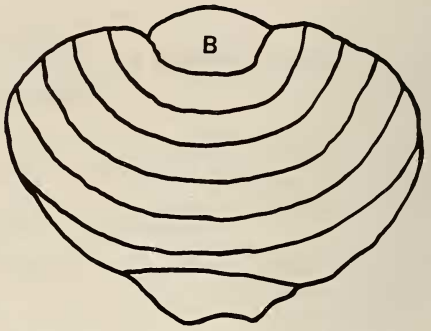

Fig. 2. Schematic drawing showing how a section of explant was compartmentalized for quantitative analysis. Lines were drawn at 50 μm intervals.

bated with monoclonal antibody to BrDU (Amersham) for 60 min and then with peroxidase-conjugated anti-mouse IgG. The reaction product was visualized with 3,3′-diaminobenzidine solution containing H_2O_2. In all explatns, different levels of sections at 8 μm intervals were mouted on a slide and stained. After confirming that the pattern of BrDU-labelling was consistent irrespective of the section levels, a section containing a maximal number of labelled cells was selected for quantitative measurement. In those cultures where the brain was co-cultivated, lines were drawn on each photomicrograph of adenohypophysial tissue at 50 μm intervals along the boundary of the attached brain (see, Fig. 2). The entire area of adenohypophysial tissue was measured by use of an image analyzer (IBAS-2000, Germany) whereas the area of each compartment was calculated by the paper weight method. Then the respective areas of the compartments were calculated based on their weight rate. In case of explants without the brain, only the entire area was measured. The frequency of labelled cells was expressed as the number of labelled cells per area.

RESULTS

In vivo observations

On day 12.5 of gestation, the epithelial walls of the adenohypophysial primordium were found to contain many BrDU-labelled cells. There was no topographical difference in the distribution of labelled cells particularly in terms of the presumptive neural lobe (Fig. 3a). On the other hand, a marked regional difference in labelling was observed in the adenohypophysial primordium on days 13.5 and 14.5. During this period, labelling was mostly confined to the upper half of the adenohypophysial tissue that faced the neural element (Fig. 3b). On day 16.5 BrDU-labelled cells were sparsely distributed throughout the adenohypophysis (Fig. 3c).

In vitro experiments

When maintained in organ culture for 1–2 days, the Rathke's lumen was found to be narrowed to varying degrees. In some cases, the lumen was obliterated completely. Identification of brain tissue was easy because great care was taken as to the orientation of explants throughout the course of experiment as already described in Materials and Methods. Most explants had no typical mass of mesenchymal tissue. Some cultures, however, were found to possess a small amount of mesenchyme at a confined area usually toward the opposite side of brain tissue (Fig. 4d).

In explants that were separated on day 12.5 and cultivated for one day with the diencephalic floor, adenohypophysial tissue was crowded with many BrDU-labelled cells. The majority of labelled cells were included in the half of explant to which brain tissue attached (Fig. 4a). Fig. 5a shows the result of quantitative data on the distribution of prolifer-

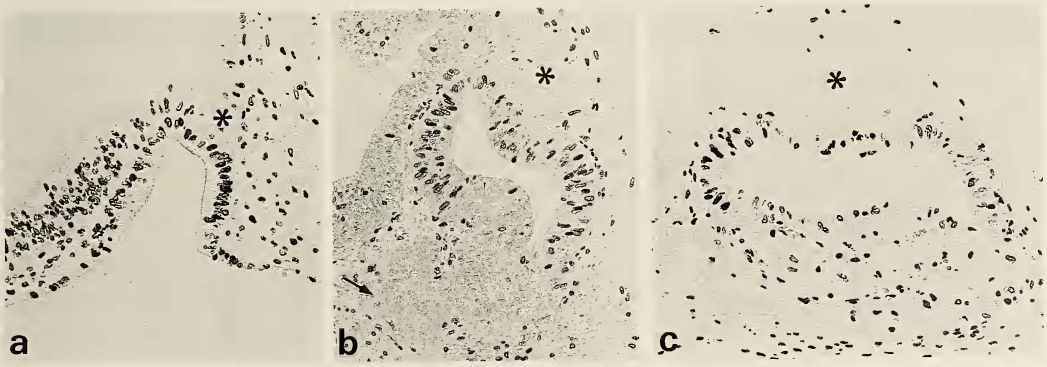

FIG. 3. Photomicrographs showing BrDU-labelled proliferating cells in the pituitary primordia of fetal rats. Asterisk indicates the presumptive neural lobe. ×105. a. On day 12.5 labelled cells are observed homogeneously. b. On day 14.5 only a few cells are labelled in the lower half of the adenohypophysial primordium. Labelling is completely lacking in the lateral lobe (arrow). c. On day 16.5 labelled cells are distributed homogeneously.

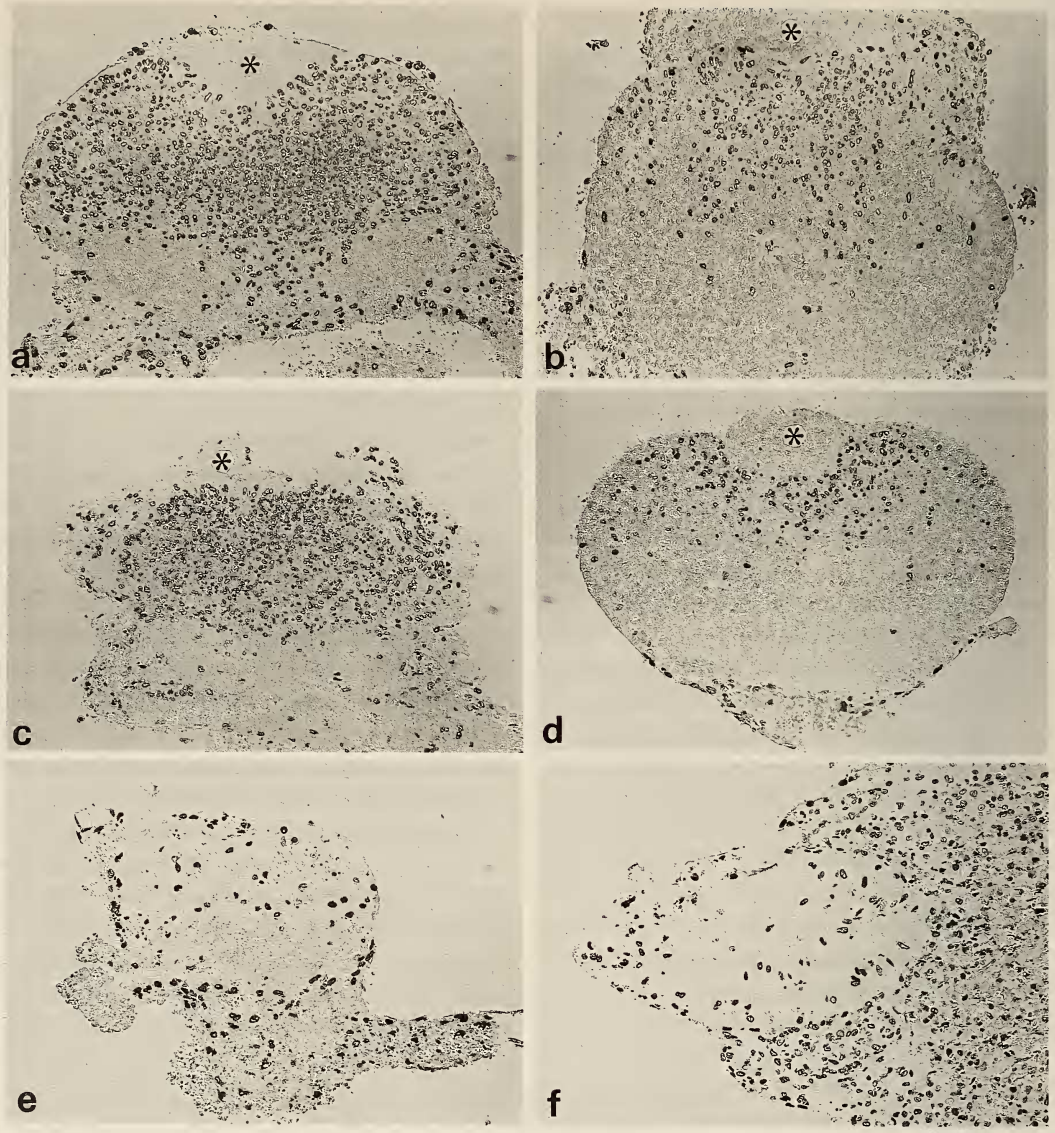

Fig. 4. Photomicrographs showing BrDU-labelled cells in culture explants of adenohypophysis removed on days 12.5 (a, b, c, e, f) and 13.5 (d) of gestation. Asterisk indicates co-cultivated brain. ×113. a. One-day explant. Labelled cells are sparsely distributed toward the opposite side of the brain. b. Two-day explant. The distribution pattern of labelled cells is essentially similar to that in a. c. One-day explant treated with collagenase before culture. d. One-day explant. Labelled cells are seen toward the brain. e. One-day explant without the brain. f. One-day explant explant co-cultivated with liver tissue after removal of the brain.

ating cells with special reference to brain tissue. The density of proliferating cells was highest toward the brain.

After removal of the diencephalic floor, the number of BrDU-labelled cells was markedly reduced (Figs. 4e, 6a). The distribution of labelled cells was inconsistent. This was also the case if the brain was replaced by liver tissue (Figs. 4f, 6a). Collagenase treatment did not affect proliferative activity of the adenohypophysial primordium (Figs. 4c, 5a).

The overall incidence of labelling became lower after 2 days in culture (Figs. 5a, 6a). Owing to such lowered activity of cell proliferation, the

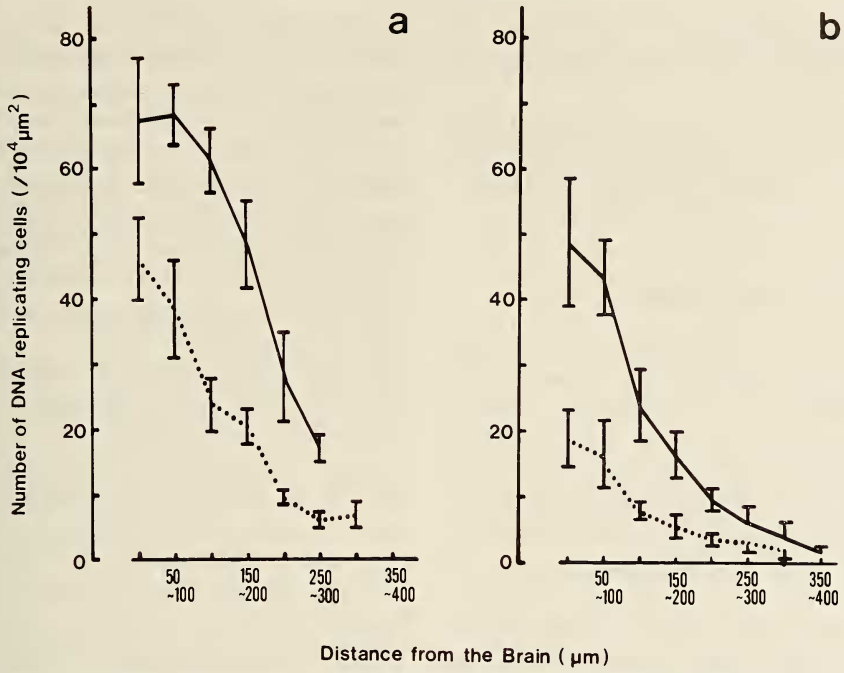

FIG. 5. Frequency of BrDU-labelled cells in adenohypophysial primordium isolated on days 12.5 (a) and 13.5 (b) of gestation. solid line, 1-day culture; broken line, 2-day culture. The vertical bars represent the mean ± SEM of 3–6 explants.

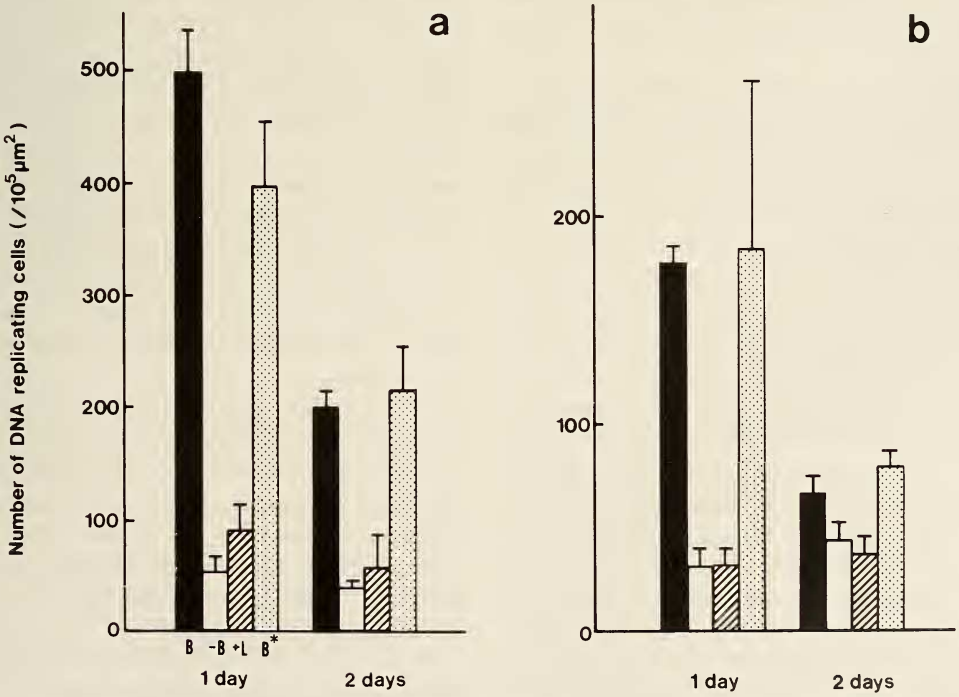

FIG. 6. Proliferative activity of adenohypophysial primordium isolated on days 12.5 (a) and 13.5 (b). B, culture with brain; -B, culture without brain; L, culture with liver; B*, culture with brain after collagenase treatment. The vertical bars represent the mean ± SEM of 3–6 explants.

characteristic distribution of BrDU-labelled cells in terms of brain was more clearly observed (Fig. 4b, 6a).

When the adenohypophysial primordium separated on day 13.5 was cultivated, results were almost similar though the incidence of labelled cells were generally lower (Figs. 4d, 5b, 6b).

DISCUSSION

Differentiation of many organs is known to be accomplished through inductive interactions between intimately associated tissues of different natures. Epithelio-mesenchymal interaction is the most well analyzed phenomenon in various species of animals. In case of the developing pancreas, Rutter and his colleagues [15, 16] have postulated that the differentiation of this organ is caused by a factor produced by the surrounding mesenchyme. This substance or "mesenchymal factor" plays a role not only in the cytodifferentiation of pancreatic cells but also in the stimulation of DNA replication of the primordial cells [17]. There is also evidence that cell proliferation precedes the final cytodifferentiation in muscle [18], bone [19] and kidney [20].

Opinion is not yet uniform as to what factor(s) is involved in cell proliferation and/or differentiation of the adenohypophysial primordium. Some investigators have postulated surrounding mesenchme is responsible for the differentiation of the adenohypohysis [21–24]. Others [5, 7–9, 25, 26] have presumed that the diencephalic floor is rather indispensable for organogenesis of this organ. Quantitative studies on the proliferative rate of adenohypophysial cells may provide a clue to this problem. Both mitotic figures [11, 12] and ^{3}H-thymidine uptake [27] were more frequently observed in the dorsal half of the adenohypophysial primordium that is in contact with the developing diencephalon. Proliferative activity of cells were generally lower in the ventral half of the adenohypophysial primordium that is surrounded by mesenchyme. In our study, such characteristic pattern of cell proliferation was observed in the adenohypophysial primordium of fetal rats on day 13.5. Ikeda and Yoshimoto [13], on the other hand, have reported that this heterogenous labelling was found as early as day 12.5 of gestation. The discrepancy between their and our observations may be due to the difference in the length of labelling time, or in the technique of DNA denaturation. BrDU, a thymidine analogue, is known to be incorporated into the cell nucleus at the S phase, which has been shown to vary according to the developmental stage of animals. It remains, therefore to be determined whether our data reflect the actual rate of cell proliferation of the developing adenohypophysis.

To date, *in vitro* evidence indicates the stimulatory effect of the diencephalic floor on the proliferation of adenohypophysial cells. Adenohypophysial tissue grew well when it was cultivated with the diencephalic floor. Removal of brain tissue, on the other hand, resulted in a marked malgrowth of explants [7, 9, 10]. These observations are in favor of the concept that the neural element rather than mesenchyme plays an essential role in the proliferation of developing adenohypophysial cells. Our results further strengthen the effect of the diencephalic floor. The proliferative activity of adenohypophysial cells was far higher in the presence of the diencephalic floor. Furthermore, proliferating cells were concentrated mainly in the region of the adenohypophysis that faced brain tissue. In the light of the present results, it seems reasonable to speculate that the developing diencephalic floor produces some unknown substance that is involved in the cell proliferation of adenohypophysial primordium. Recent studies have shown the presence of platelet derived growth factor (PDGF) in the neurohypophysis [28], or insulin like growth factor (IGF)-II in the developing diencephalic floor [29]. Other than these growth factors, ACTH-related peptide has been shown in the hypothalamus of fetal rats as early as day 12 [30]. In view of a mitogenic effect of ACTH in myogenic cells [31], this adenohypophysial hormone may also be taken into consideration. Further studies are needed to elucidate the exact nature of the neural influence on the developing adenohypophysis.

In this study, BrDU-labelled cells became less dense as the duration of organ culture. This is partly due to the culture condition employed: namely, a very low concentration of serum added

(0.1%). It must be stressed, however, that the number of BrDU-labelled cells in the developing rat adenohypophysis has been shown to decrease *in vivo* as well, especially after day 14.5 of fetal age [13]. Their results agree with those of our study. Thus, the stimulative effect of the diencephalic floor on adenohypophysial cell proliferation probably becomes less important as development proceeds.

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